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A *strawberry notch* homolog, *let-765*/*nsh-1*, positively regulates *lin-3*/*egf* expression to promote RAS-dependent vulval induction in *C. elegans*

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ABSTRACT

The specification and patterning of vulval precursor cells (VPCs) in *Caenorhabditis elegans* is achieved using a conserved EGFR/RAS signaling pathway that is activated by the ligand *lin-3*/EGF, which is secreted by the neighboring somatic gonad. Previous work has demonstrated that the expression of *lin-3* must be tightly regulated to ensure that only three of six equivalent VPCs are induced to differentiate into the mature vulva. Here, we have identified a novel regulator of EGFR/RAS signaling, *let-765*/*nsh-1*, that functions upstream of the pathway to promote vulval induction. *let-765* encodes a conserved DExD/H box helicase protein and is the *C. elegans* ortholog of *Drosophila strawberry notch*. By investigating genetic interactions between *let-765* and RAS pathway genes as well as with synthetic multivulva (synMuv) genes, we have demonstrated that *let-765* positively regulates the RAS pathway and antagonizes synMuv activity at the level of *lin-3*/EGF. In support of these proposals, we found that LET-765 is required for producing wild-type levels of *lin-3* mRNA. Mutations in *let-765* result in pleiotropic phenotypes that imply its function must be required in multiple developmental processes and, together with data presented here, suggest that LET-765 promotes the expression of diverse targets, potentially through interactions with transcriptional activator or repressor complexes.

Introduction

The specification of distinct cell fates is often achieved through the activation of specific intercellular signaling pathways by growth factors whose expression must be spatially and temporally controlled to ensure accurate responses. Typically, a target cell expresses an excess of receptors such that the availability of its ligand is rate-limiting for pathway activation. Overall, for a signal to elicit the desired response, the intensity and duration of ligand expression must be sufficient to meet its threshold concentration, and yet the signal response must be restricted to prevent excessive signaling (Freeman and Gurdon, 2002). Excessive activation of a signaling pathway can have broad deleterious effects, thus prompting a requirement for antagonistic regulation.

In *Caenorhabditis elegans*, an epidermal growth factor receptor (EGFR) mediated RAS pathway is used in development and differentiation of multiple types of tissues (Sundaram, 2006). Loss of function mutations in core pathway components leads to early larval lethality. Arrested L1 stage larvae exhibit rod-like morphology and fill with fluid, thought to result from a defect in osmoregulation, (Aroian and Sternberg, 1991; Nelson et al., 1983). Furthermore, EGFR/RAS signaling is also required for spicule development in the male tail (Chamberlin and Sternberg, 1994), vulval induction (Aroian and Sternberg, 1991), differentiation of the vulval-uterine connection (Chang et al., 1999) and P12 cell specification in the posterior ectoderm (Jiang and Sternberg, 1998). In *C. elegans*, the development of the vulva has been extensively studied as a model for understanding how multiple signaling pathways and their cues can be integrated to specify a variety of cell fates (Horvitz and Sternberg, 1991; Sternberg, 2005; Yoo et al., 2004).

Vulval development is achieved by the action of intercellular signaling pathways that direct cell fate specification events via the coordinated regulation of numerous transcription factors. Vulval precursor cell (VPC) specification is directed by signals from the EGFR, Notch and Wnt pathways through both synergistic and antagonistic interactions, to establish a precise order of events that effect an invariant pattern of development to form the mature organ. Six ventral epithelial cells, P3.p through P8.p, form the vulval equivalence group, and are competent to acquire one of three vulval cell fates, 1°, 2°, or 3°. Vulval development is induced by an EGF-like growth factor, LIN-3, that is secreted by the gonadal anchor cell (AC) (Hill and Sternberg, 1992). Each of the VPCs (P3.p through P8.p) expresses the EGF-receptor homolog LET-23 (Aroian et al., 1990) and are capable of receiving the growth factor signal to activate a RAS/MAPK signaling pathway that specifies vulval cell fates via LET-60/RAS (Beitel et al., 1990; Han and Sternberg, 1990), MEK-2 (Kornfeld et al., 1995; Wu

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et al., 1995) and SUR-1/MPK-1/MAPK (Lackner et al., 1994). Under typical physiological conditions P6.p, the cell closest to the AC, acquires the 1° fate, resulting from high levels of RAS pathway activity. In response to the LIN-3/EGF inductive signal, P6.p produces a lateral signal that activates LIN-12/NOTCH in adjacent cells, P5.p and P7.p (Sternberg and Horvitz, 1989; Yoo et al., 2004) to promote the specification of 2° cell fates (Greenwald et al., 1983; Simske and Kim, 1995). RAS and Notch signaling levels are low in distal VPCs, producing the default, 3° fate. These cells divide once and fuse with the hypodermal syncytium (hyp7), although occasionally P3.p will fuse with hyp7 without dividing. Experiments with LIN-3/EGF indicate that the growth factor signal acts in a graded manner (Katz et al., 1995). As a result of this gradient, P6.p receives the greatest amount of the signal, acquiring the 1°cell fate, whereas intermediate amounts of signal can specify 2° cell fates in P5.p and P7.p, possibly in cooperation with Notch signaling (Kenyon, 1995).

Mutations that reduce the function of the EGFR/RAS pathway components result in the induction of less than three vulval precursor cells and a vulvaless (Vul) phenotype. Activating mutations or over expression of *lin-3/egf* cause distal cells to be induced and produce a multivulva (Muv) phenotype. Not surprisingly, there exists an antagonistic mechanism, which ensures that only three cells can adopt vulval fates and produce a functional vulva. This is accomplished in part by the synthetic multivulva (synMuv) genes which antagonize EGFR/RAS signaling (reviewed in (Fay and Yochem, 2007). The synMuv genes are classified into two principal groups—synMuv A and synMuv B—based on the observation that single or double mutants from one class develop a wild-type vulva, but an A-B double mutant produces a strong Muv phenotype; the synthetic interaction implies that these genes act in two redundant processes to inhibit vulval induction (Ferguson and Horvitz, 1989). The molecular functions of synMuv A genes have not been established, but they have been predicted to play a role in transcriptional regulation (Davison et al., 2005; Huang et al., 1994). By homology comparisons to *Drosophila* and mammalian proteins, the large synMuv B class includes proteins predicted to act as transcription factors and chromatin modifiers, including homologs of the mammalian Rb/E2F complex (LIN-35/Rb and EFL-1/DPL-1 E2F) (Ceol and Horvitz, 2001; Lu and Horvitz, 1998; Thomas et al., 2003), the nucleosome remodelling and deacetylase (NuRD) complex (Solari and Ahringer, 2000; Unhavaithaya et al., 2002; von Zelewsky et al., 2000), histone methyltransferases (Andersen and Horvitz, 2007), and a DP/Rb/MuvB (DRM) complex (Harrison et al., 2006). The homology to Rb/E2F and NuRD complexes suggests that the synMuv proteins function in transcriptional repression. Specifically, as a result of *lin-3/egf* being identified as a transcriptional target of many synMuv genes (Andersen and Horvitz, 2007; Andersen et al., 2008; Cui et al., 2006a), it has been proposed that synMuv genes function to repress *lin-3* expression thus inhibiting ectopic vulval induction. A number of genes have been identified that function in various aspects of chromatin remodeling and transcriptional regulation and are suppressors of the synMuv phenotype (Andersen et al., 2006; Cui et al., 2006b), underscoring the potential complexity of interactions between regulatory complexes that modulate gene expression.

DExD/H box helicases are a large family of proteins that function in many aspects of RNA metabolism including transcription, mRNA splicing and export, RNA stability, and translation (Linder, 2006; Rocak and Linder, 2004; Schwer, 2001; Tanner and Linder, 2001). Proteins in this family contain motifs which, when combined, compose a helicase domain and residues required for ATP-dependent nucleic acid binding activity. Although formally thought of as ATP-dependent RNA helicases, due to early examples of this activity and their homology to DNA helicases, these proteins are now thought to be involved in multiple aspects of RNA synthesis and processing, extending to potential RNPase activity (Linder, 2006; Rocak and Linder, 2004; Schwer, 2001). In addition, there is emerging evidence that some DExD/H proteins possess a helicase-independent ability to interact with transcriptional activators and repressors (Wilson et al., 2004). It has been proposed that they act as bridging factors to recruit transcriptional activators to the initiation complex or provide stability to the complex by interacting with multiple components (Fuller-Pace, 2006).

In this study, we present the identification of a DExD/H box helicase gene, *let-765*, which is the *C. elegans* ortholog of *Drosophila strawberry notch* (*sno*). Early studies in *Drosophila* revealed that mutations in *sno* lead to phenotypes similar to those of Notch mutants and that Sno interacts genetically with proteins that regulate the transcription of Notch target genes (Majumdar et al., 1997; Nagel et al., 2001). More recently, a role for Sno during eye development has been reported where it functions to promote the expression of the Notch ligand, Delta, in R cells (Tsuda et al., 2002) This, together with the proposed role for DExD/H box proteins in transcriptional regulation, led us to hypothesize that *let-765* may play a role in promoting gene expression. We have cloned *let-765/nsh-1* and used mutant alleles and RNAi to assess its loss of function phenotype with the aim of obtaining insight into its potential function. When *let-765* activity was reduced, we observed a decrease in vulval induction and a protruding vulva phenotype, suggesting that LET-765 is required for proper vulval development. An examination of genetic interactions between *let-765* and the EGFR/RAS pathway demonstrated that *let-765* is required for wild type vulval induction and for producing the synMuv phenotype. Subsequently, we found that the level of *lin-3/egf* transcripts were reduced in wild type and in synMuv mutants as a consequence of reduced *let-765* activity, supporting a role for LET-765 in promoting *lin-3/egf* expression. *let-765* null mutants exhibit early larval arrest with a rod-like lethal phenotype, suggesting that this gene may also interact with the EGFR/RAS pathway to promote viability. Our results reveal that *let-765/nsh-1* promotes vulval induction upstream of the EGFR/RAS pathway by positively regulating *lin-3/egf*. This identifies a new positive regulator of *lin-3/egf* and a novel role for a *strawberry notch* homolog.

Materials and methods

General methods and strains

C. elegans were cultured using standard methods (Brenner, 1974) and experiments were performed at 20 °C unless otherwise noted. N2 (Bristol) was used as wild type. Additional information regarding alleles can be found at www.wormbase.org.

LGI: *dpy-5(e907)*, *lin-35(n745)*, *lin-53(n833)*, *pry-1(mu38)* (Gleason et al., 2002), *hT2*(*qIs48*) (I,III) LGII: *let-23(sa62gf)* (Katz et al., 1996), *lin-8 (n111), lin-31(n301, n1053), rrf-3(pk1426)* (Simmer et al., 2003); LGIII: *dpy-17(e164)*, *dpy-19(e1259)*, *let-765(s2575, s2630)* (this study), *nsh-1 (ok2058)* (C. elegans gene knockout consortium), *lin-9(n112), lin-12 (n137), lin-36(n766), unc-32(e189), sDf125(s2424), sDp3*(III,f), LGIV: *dpy-20(e1282)*, *let-60(sy100, n1046, ga89)* (Beitel et al., 1990; Eisenmann and Kim, 1997; Han and Sternberg, 1991), *lin-1(e1777) lin-3(e1417), nT1(m435)* (IV,V) LGV: *him-5(e1490),* LGX: *bar-1(ga80), lin-15A(n767), lin-15B(n744), lin-15AB(n765)*.

Extrachromosomal or integrated arrays:

arIs92[*egl-17*::*nls*::*cfp*::*lacZ*] (Yoo et al., 2004), *gaIs36*[hs-*mpk-1*, IF1alpha-Dmek-2] (Lackner and Kim, 1998), *qIs56*[*lag-2::gfp*; *unc-119 (+)*] (Siegfried and Kimble, 2002), *saIs14*[*lin-48::gfp*, *unc-119(+)*] *sEx718*[F20H11, *pCes1943*], *sEx587*[F27B3, *pCes1943*], *sEx996* [F20H11_KpnI, F22E10.4::*gfp*, *pCeh361*], *sEx1018*[F20H11_XhoI, F22E10.4::*gfp*, *pCeh361*], *sEx1800*[*let-765p*::*nls*::*gfp*; *pCeh 361*], *sEx1729* [*pdpy-7*:F20H11.2, *pCeh361*, *myo-2:gfp*], *sIs1637*[WRM0629dG03::*gfp*, *pCeh361*, F22E10.4::*rfp*], *syIs1*[*lin-3*(+)] (Hill and Sternberg, 1992), *syIs103*[*lin-11::gfp*, *unc-119(+)*] (Gupta and Sternberg, 2002), *zhIs4*[*lip-1::gfp*] (Berset et al., 2001).

Identification and cloning of let-765 (s2575) and (s2630)

s2575 and *s2630* were mapped to the left arm of Chromosome III, in the region defined by the deficiency *sDf125* and duplication *sDp8*. The lethal phenotype of *s2575*, and later *ok2058*, was rescued by cosmid F20H11 (10 ng/μL). Cosmid DNA was digested with either *Xho*I or *Kpn*I and re-ligated. Rescues with the overlapping cosmid subclones identified the gene model corresponding to *let-765* as either F20H11.2 or F20H11.6. *let-765* genomic DNA was amplified from homozygous mutant animals and sequenced across the appropriate region. Mutations in F20H11.2 were confirmed by sequencing duplicate PCR products on both strands and comparing the results with N2.

cDNA clones yk1729e03 and yk536c4 (a gift from Y. Kohara, National Institute of Genetics, Mishima, Japan) were sequenced and compared with the predicted exon–intron structure. Additional RT-PCR experiments confirmed the coding regions and splice sites and included the extra exon identified at the 3' end of yk536c4. A second reaction, using an SL2 primer, identified an additional four exons at the 5' end, overlapping with the upstream gene F20H11.6, and redefined the corresponding splice junctions (genbank: GU997622). We did not obtain any support for the previously annotated F20H11.2 transcript. BLAST identified the closest ortholog in *Drosophila* as NP_001096968.1 and mammalian orthologs (mouse NP_001074672.1 and human NP_060653.2).

Transgenic constructs

The *let-765p*::gfp transcriptional promoter:GFP construct was made using PCR fusion (Hobert, 2002) and primers A (5'- CTC ATT CGC TTC CCA TTA ATC A-3'), A* (5'-CGC TTC CCA TTA ATC AAC CTT A-3'), and B (5'-agt cga cct gca ggc atg caa gct CCG AGA GTA TGT CAT CGA TTT-3'). Linear PCR products were injected at 50 ng/μL. A *dpy-7p*::GFP construct was generated by amplifying a 216 bp region of the *dpy-7* promoter using primers pD7-1 (5'-GGA CTT AAG CTT CCA CGA TTT CTC GCA ACA CAT-3') and pD7-2 (5'-GGA TTA CTG CAG AAA AAG AAC AGG GTG TGA TAA ATG-3') and cloning the product into the *Hin*dII–*Pst*I sites in pPD95.75. The *Xma*I–*Spe*I fragment containing the GFP coding region and *unc-54* 3'UTR was replaced with a PCR amplified genomic clone of *let-765* using primers CS100 (5'-GAT ATC CCG GGT CCA GTA GGA ATG GCT G-3') and CS101 (5'-GGT AGA CTA GTC ACA AAT GTA TCG AAA GGG AAA-3') generating *dpy-7p*:LET-765. *dpy-7p*:LET-765 was injected at 10 ng/μL and 3 ng/μL.

pCes1943 (100 ng/μL) (Janke et al., 1997), *pCeh361* (100 ng/μL) (Thacker et al., 2006), F22E10.4::*gfp* (50 ng/μL), F22E10.4::*rfp* (50 ng/μL), or *myo-2::gfp* (20 ng/μL) were used as a co-transformation marker.

Recombineered fosmid: GFP

A PCR amplified cassette containing the GFP coding region was inserted into fosmid WRM0629dG03 by the method described in (Dolphin and Hope, 2006; Stavropoulos and Strathdee, 2001). Primers used were: 765 RT-L (5'-CCA CAA CTA ATG GAA TGG TCA TAC AAG CCA AGA CAC CGG GGG CTG GCG TTt cgc tgt cga gat atg acg gtg-3'), 765 RT-R (5'-GGG CCT CCC GAT GGG AAA CTT TGT TGG GAT TGC ATA TGT CTT GCC TGA ATg atg ata agc tgt caa aca tga g-3'), 765 gfp-L (5'-CCA CAA CTA ATG GAA TGG TCA TAC AAG CCA AGA CAC CGG GGG CTG GCG TTa tga gta aag gag aag aac ttt tc-3'), 765 gfp-R (5'-GGG CCT CCC GAT GGG AAA CTT TGT TGG GAT TGC ATA TGT CTT GCC TGA ATt ttg tat agt tca tcc atg cca tgt g-3').

RNAi feeding experiments

RNAi by feeding was performed as described in Kamath et al. (2003) with the following modifications: worms were grown on NGM lite plates containing 50 μg/mL carbenicillin, 12.5 μg/mL tetracycline and 4 mM IPTG. L4 animals were plated on HT115 bacteria expressing F20H11.2 dsRNA or empty vector control (pPD129.36) and incubated at 23 °C for 24–30 h. P_o hermaphrodites were removed and progeny were allowed to develop. Experiments were repeated a minimum of two times to confirm phenotypic results.

Phenotypic analysis and vulval induction assays

Arrested rod-like lethal progeny of RNAi-treated hermaphrodites were scored by allowing mothers to lay eggs for 24–30 h and progeny L1 arrested animals were counted and removed. Vulval induction was scored at the L4 stage using Nomarski optics as described (Sternberg and Horvitz, 1986). The number of VPCs that acquired a 1° or 2° fate was counted in each animal. The induction index was calculated by dividing the total number of 1° and 2° induced cells by the total number of animals scored. Animals were scored as Muv if any of P3.p, P4.p, or P8.p were induced and Vul if one or more of P(5–7).p was not induced. Statistical analysis was performed using a Mann–Whitney test.

Microscopy

Analysis and manipulation of fluorescent transgenics and *hT2* balanced mutants was performed using a Zeiss Stemi SVC11dissecting microscope equipped with epiflourescence. Images were collected using a Zeiss Axioscop equipped with epiflourescence and a QImaging camera or a WaveFX spinning disc confocal microscope (Zeiss Axio Observer microscope and Hammamatsu EMCCD camera) using Improvision software.

Quantitative real-time RT-PCR (qRT-PCR)

Synchronized L1 larvae of the various genotypes were grown on *let-765* RNAi or empty vector control until early L3, estimated by the degree of gonad development. Worms were washed 3–5 times in M9 and frozen in liquid nitrogen. Total RNA was isolated using Trizol (Invitrogen) followed by treatment with DNaseI and purification. cDNA was prepared from 1 μg of total RNA using a combination of oligo $dT_{(17)}$ and random hexamer primers and Superscript II reverse transcriptase (Invitrogen) as per manufacturer's instructions. Multiple biological replicates were collected and cDNA reactions pooled. Each qRT-PCR reaction contained 50 ng of RT products, 10 μL of 2× SYBR Green Supermix (Biorad), and 1 μM of each primer. qRT-PCR reactions were run in triplicate on a Biorad MyIQ Real-time thermocycler. Data were normalized using *rpl-19* and the relative fold change was calculated using the ΔΔCt method. All primers were tested on serial dilutions and primer efficiency calculated.

Results

let-765 is encoded by F20H11.2/nsh-1

The *let-765* locus was initially defined by two mutations, *s2575* and *s2630*, obtained from a screen for essential genes on the left arm of Chromosome III (Stewart et al., 1998). Using transformation rescue, we discovered that cosmid F20H11 rescues the lethal phenotype of *s2575*, whereas the overlapping cosmid F27B3 does not, leaving six candidate genes that could encode *let-765*. With the expectation that *let-765* RNAi would reflect the essential nature of the gene, only F20H11.3, F20H11.6, and F20H11.2 remained as the best possibilities—each of these gives embryonic lethal RNAi phenotypes when administered by injection. Further rescue experiments with F20H11 subclones and overlapping fosmid clones showed that *let-765* is encoded by F20H11.2, annotated as *n*otch *s*ignaling pathway *h*omolog-1 (*nsh-1*). To add further support to the identity of *let-765*, genomic DNA from homozygous mutant animals was sequenced throughout the F20H11.2 region and a single base substitution was identified for each allele. The mutation in *s2630* converts a conserved tryptophan to a stop codon that would eliminate

the C-terminal half of the predicted protein, whereas *s2575* contains an alteration of C to T in a conserved residue upstream of the previously annotated start codon, which we subsequently identified as part of the F20H11.2 coding sequence (Fig. 1).

In addition to our existing alleles, the *C. elegans* Gene Knockout Consortium generated a deletion allele (*ok2058*) in *let-765*/*nsh-1* that removes 1541 bp and inserts a C at the 5' end of the deletion site—the deletion and frameshift result in a truncation of the predicted protein. *ok2058* homozygotes display the same early larval lethality as *s2575* and *s2630* and all three alleles fail to complement one another, verifying that they are allelic, thus we have identified *let-765* as F20H11.2/*nsh-1*.

We determined the sequence of the F20H11.2 transcript by RT-PCR and by sequencing two available cDNAs (yk1729e03 and yk536c4). All exons and splice junctions were confirmed including an additional exon at the 3' end that was also found in the yk536c4 sequence. The 5' end of the gene was identified by SL2 RT-PCR and includes four additional exons that had been annotated as the upstream gene F20H11.6 (WS203). As a result of this upstream sequence being added to the existing F20H11.2 gene model, the *s2575* mutation would be classified as an arginine to a STOP, and better correlates with the strong loss of function phenotype. Translation of the updated open reading frame results in a protein of 1866 amino acids in length.

F20H11.2 encodes a DExD/H box helicase (Cordin et al., 2004; Tanner and Linder, 2001)—a family of proteins defined by a set of characteristic motifs including the DExD/H box. LET-765 contains an Asp–Glu–Cys–His (DECH) in place of the signature DEAD box "Walker B" motif and an analysis of the protein sequence using SMART (Schultz et al., 2000) and Pfam (Finn et al., 2008) identified a C-terminal helicase domain. Closer inspection of the protein sequence revealed that LET-765 shares with other helicase proteins nearly all of the conserved residues that are required for their enzymatic activity (Cordin et al., 2004; Linder, 2006; Tanner and Linder, 2001). However, the additional N-terminal sequence predicted from the cDNA does not contain any characterized domains and BLAST results indicated it is likely to be nematode specific. In summary, the structure of LET-765 suggests that it may be involved in RNA metabolism or the regulation of gene expression.

LET-765 is the ortholog of strawberry notch, a gene involved in transcriptional regulation of Notch and EGFR target genes

LET-765 is the *C. elegans* ortholog of *Drosophila strawberry notch* (*sno*) and mammalian *SBNO1*. It is well conserved with its homologs throughout the functional domains, with greater divergence at the terminal portions of the protein, which is a common feature among DExH/D box family genes (Cordin et al., 2006) (Fig. S1). BLAST searches revealed that mammalian genomes contain two *sno* homologs, while *Drosophila* and *C. elegans* each have a single member. In addition, *sno* proteins are found within many multicellular organisms, but yeast or bacterial homologs have not been identified.

Drosophila Sno was first characterized as a component of Notch-mediated processes and, in one such example, cooperates with vestigial (vg) and scalloped (sd) to activate the transcription of cut (ct) at the wing margin (Majumdar et al., 1997; Nagel et al., 2001). This occurs downstream of Su(H), which positively affects transcriptional activity in this context. On the other hand, a role for Sno in promoting Notch signaling during eye development, downstream of the EGFR/RAS pathway, has also been identified. The specification of cell fates in the *Drosophila* eye requires the sequential action of EGFR/RAS and Notch signaling pathways in a manner analogous to that which occurs during *C. elegans* vulval development (Sundaram, 2005; Voas and Rebay, 2004). An EGFR/RAS pathway is activated in R cells, leading to the expression of Delta (Dl) which signals to adjacent cells via the Notch receptor. The Notch pathway then induces the expression of cone cell-specific transcription factors, establishing the cone cell fate. Sno appears to promote Dl expression through antagonism of Su(H)-mediated transcriptional repression. Genetic

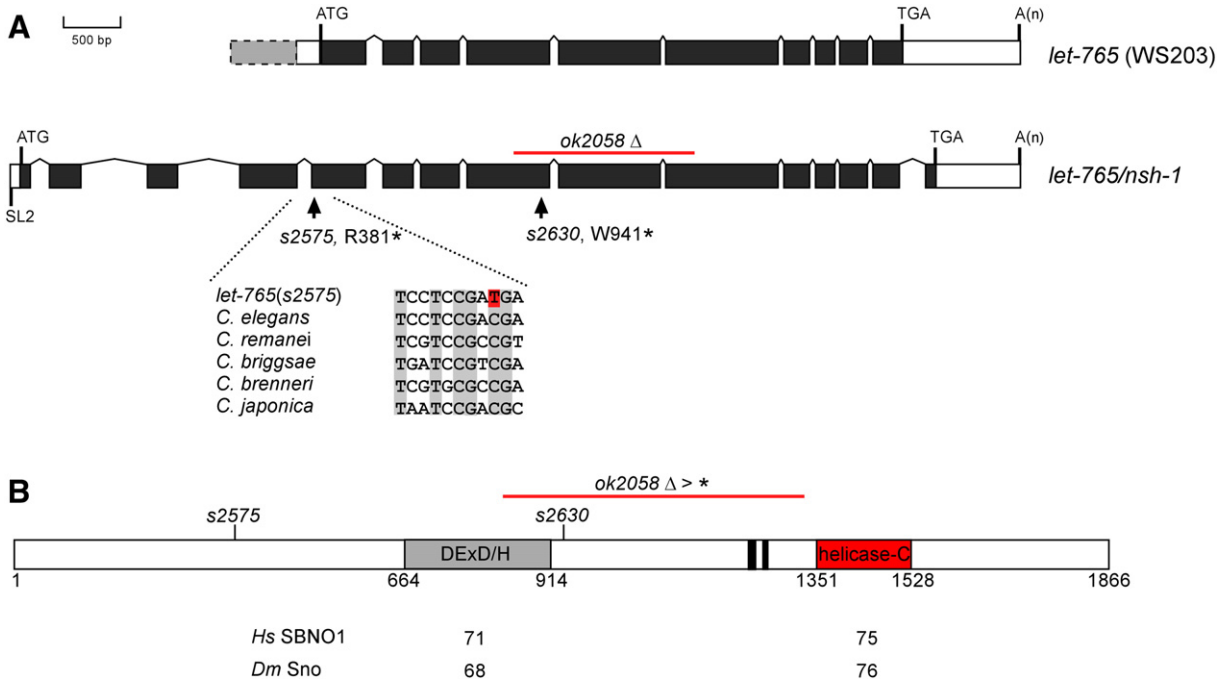

Fig. 1. *let-765* encodes a DExD/H box protein. (A) Gene structure of *let-765*. Exons are shown as black boxes, 3' and 5' untranslated regions are shown as white boxes. In *let-765* (WS203), the predicted upstream gene is dashed and shaded grey. Translational start and stop sites and polyadenylation sites are indicated and the *s2575* and *s2630* mutations and *ok2058* deletion are shown. The Wormbase prediction is labeled *let-765* (WS203) and structure based on our SL2 RT-PCR is marked *let-765*/*nsh-1*. The *s2575* mutation affects a residue that is conserved among *Caenorhabditis* species. (B) Schematic representation of the domain structure of the LET-765 protein with the location of the mutations marked. It is the *C. elegans* homolog of *Drosophila strawberry notch* and related mammalian SBNO proteins. The DExD/H and helicase C domains are well conserved between species—the percent identity when compared to *Homo sapiens* SBNO1 and *Drosophila* Sno is noted below. A full alignment with *Drosophila* and *H. sapiens* sequences is shown in Fig. S1.

and molecular evidence suggests that Sno cooperates with the F-box/WD40 protein ebi to disrupt a Su(H)/SMRTER repressor complex, leading to nuclear export and degradation of SMRTER (Tsuda et al., 2002). A physical interaction between Sno and Su(H) has been demonstrated, lending further support to this hypothesis. Of further interest, in mammals a strawberry notch protein, SBNO2, has been studied in the context of the anti-inflammatory response. SBNO2 is up-regulated by STAT3 dependent IL-10 signaling in macrophages and exhibits repressive activity for transcriptional targets of NF-κB (El Kasmi et al., 2007). Evidence of a role for strawberry notch family proteins in transcriptional regulatory processes seems to be emerging, with the molecular context determining whether they contribute in an activating or repressive fashion.

let-765/nsh-1 is broadly expressed in nuclei throughout development

In order to observe the expression pattern of *let-765*, we first generated a transcriptional reporter construct containing 3 kb of sequence upstream of the revised translational start site for the gene. This was fused to a green fluorescent protein (GFP) sequence containing a nuclear localization signal (nls). The *let-765p*::*nls*::*gfp* transgene (*sEx1800)* was expressed throughout larval development in the excretory system, anterior neurons, and the hypodermis. Furthermore, strong GFP expression was observed in the somatic gonad during the L3 and L4 stages (Fig. 2B, D). The pattern observed in *sEx1800* transgenics may not provide a complete profile of *let-765* expression, however, since F20H11.2 is predicted to reside in an operon and full expression may require the input of additional upstream control elements. With this in mind, homologous recombination was used to insert a cassette containing the GFP sequence into a rescuing fosmid clone, generating a translational fusion product, LET-765::GFP (*sIs1637*). GFP expression was observed in nearly all cells of the embryo from about the 100 cell stage. In larvae and adults, GFP was detectable in neurons, hypodermal cells and the seam cells, the excretory system, and intestinal cells. We noted expression in the vulval precursor cells and their descendents during mid-larval stages and strong somatic gonadal expression in the early L3 stage through to adult, similar to that seen with *sEx1800* (Fig. 2G–H). GFP expression was visible in the nucleus, but restricted from the nucleolus in all expressing cells, consistent with a role in RNA metabolism or the regulation of gene expression.

The fosmid::GFP transgene rescued all three *let-765* alleles to a sterile adult, reflecting silencing of transgene activity in the germline. However the lack of mature oocytes or embryos in the proximal gonad implies that *let-765* is required within the germline for its survival and/or maturation. In addition, we found that hypodermal expression of an F20H11.2 genomic clone under control of the minimal *dpy-7* promoter (Bulow et al., 2004; Gilleard et al., 1997) was sufficient for partial rescue of *ok2058* and *s2575* mutants, indicating that *let-765* expression in this tissue can provide its essential activity. The *dpy-7p*::LET-765 transgene also rescued the early larval lethality to a sterile adult phenotype and, interestingly, animals exhibited a protruding vulva indicating that *let-765* activity outside of the hyp7 syncytium promotes proper vulval morphogenesis. The rescued larvae were sickly with defects in body morphology including a slightly dumpy appearance, which made it difficult to accurately assess the level of vulval induction. Nonetheless, overall vulval development and morphogenesis was clearly compromised.

Reduced let-765/nsh-1 activity leads to defects in excretory duct cell development

To gain insight into *let-765* function, we began by examining the phenotype of *let-765* mutants. Hermaphrodites homozygous for either *s2630* or *ok2058* arrest near the L1/L2 molt, while *s2575* larvae survive slightly longer, arresting during the L2 stage. Homozygous mutant larvae subsist for several days after arrest, but exhibit morphology that suggests defects in osmoregulation, specifically a rod-like posture and fluid filled body cavity. The *let-765* larval arrest

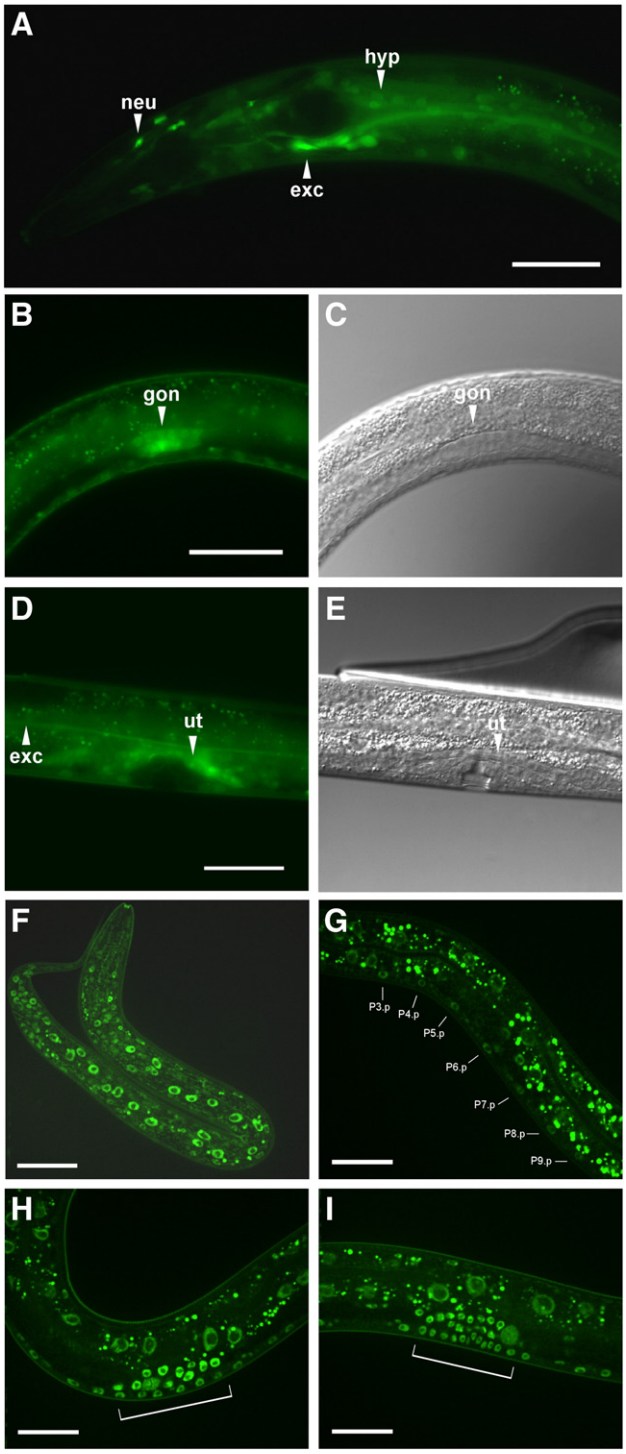

Fig. 2. GFP expression using transcriptional and translational reporters. A transcriptional *let-765p*::*nls*::GFP construct expresses in hypodermis (hyp), neurons (neu), and the excretory system (exc) throughout development (A). GFP is observed in the anchor cell and somatic gonad (gon) from mid-L2 (B-C) through L4 (D-E) where it can be seen in the developing uterus. The translational fusion, LET-765::GFP, is expressed in nearly all cells throughout larval development. An L1 stage larva (F) and Pn.p cells during the L2 stage (G). In an early L3 hermaphrodite, strong expression is visible in the somatic gonad, with continued expression in the gonad and developing VPCs at the Pn.px stage (H) and later in L3 at the Pn.pxx stage (I). Pn.p cells are labeled and VPC daughters are bracketed. Images in (C, E) are Nomarski of corresponding fluorescent images (B, D). Scale bar: 25 μm.

phenotype is fully penetrant and recessive, and placing *s2630* or *ok2058* over the deficiency *sDf125* did not affect the timing of arrest, indicating that these two alleles severely reduce, if not eliminate *let-765* function. From this we conclude that the *let-765* null phenotype is late L1-larval arrest when zygotic activity is lost and animals obtain a wild-type maternal contribution.

The rod-like morphology exhibited by arrested *let-765* mutants resembles the loss of function phenotype which accompanies mutations in *let-60*/RAS and other core RAS pathway components (Sternberg and Han, 1998; Sundaram, 2006) (Fig. S2). The *let-60*/RAS larval arrest phenotype is thought to result from a failure to specify the excretory duct cell (Sundaram, 2006; Yochem et al., 1997). The excretory duct functions to connect the excretory cell body, which provides osmoregulatory function in the nematode, to the external environment via the pore cell. To investigate whether the phenotype observed in *let-765* mutants results from a similar cell specification defect, we assayed the expression of *lin-48::gfp* (*saIs14*) in the excretory duct cell (Johnson et al., 2001). 10% of dying *ok2058* homozygotes ($n=35$) were lacking duct cell expression, similar to that seen in *let-60* null mutants, implying that the cell was missing or mis-specified (Tiensuu et al., 2005) (Fig. 3D). The remainder of *ok2058* L1 larvae examined did express GFP upon arrest although, in these animals, the cell morphology was abnormal. The excretory duct cell body was present, but the duct was missing or tracked away from the body wall (Fig. 3F). From this, we propose that *let-765* contributes to duct cell specification or to the functional properties of the duct cell and excretory system however, in some *let-765* mutants, the maternal contribution may be sufficient to partially rescue defects in cell specification.

Since the larval lethality of *let-765* mutants precluded observation of later developmental stages, we used RNAi by feeding to assess a reduction-of-function (*rf*) phenotype (Kamath et al., 2003; Timmons et al., 2003). Experiments using the RNAi-hypersensitive strain *rrf-3* (*pk1426*) (Simmer et al., 2003) resulted in a significant proportion of rod-like L1 lethal progeny (46%; $n=183$). Animals that escaped larval lethality exhibited slow growth, gonad migration defects, and a protruding vulva phenotype (Fig. 4C). In addition, nearly all larvae that developed to adulthood were sterile, with only rare cases (< 5%) of embryos present in the uterus. *let-765* RNAi-treated males had variable tail defects including underdeveloped club-like tails, spicule defects, and missing rays (Fig. 4E–F). As the rod-like lethal arrest phenotype, together with many of the RNAi phenotypes, strongly resemble those seen in animals with compromised EGFR/RAS pathway activity, it raised the possibility that *let-765* may function in cooperation with the pathway to promote development and viability.

LET-765/NSH-1 promotes vulval induction via the EGFR/RAS pathway

We opted to look closer at vulval induction and exploit its utility for characterizing genetic interactions, in order to examine the potential connection between *let-765* and the EGFR/RAS pathway. To begin with, using *let-765* RNAi, we examined the vulval cell lineage at the early L4 stage and found that 30% of the animals displayed less than wild-type induction ($n=27$), with an average of 1.1 vulval precursor cells (VPCs) induced in these animals compared to wild type (Fig. 4A–B). This implied that, in wild type animals, *let-765* functions to promote vulval induction. To investigate this putative

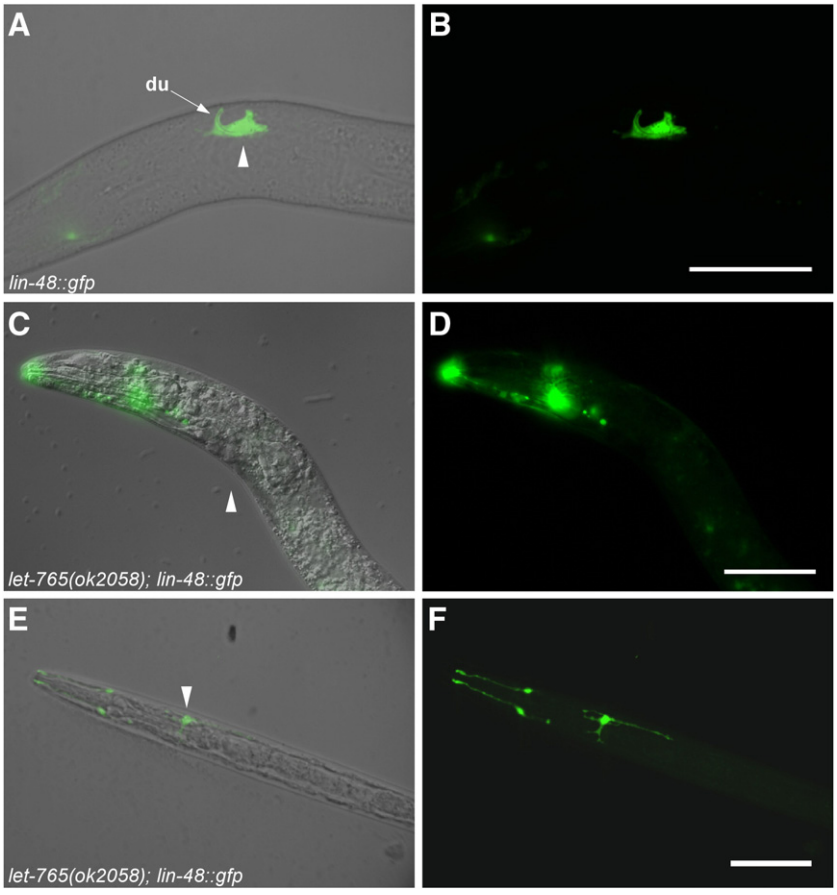

Fig. 3. *let-765* is required for excretory duct cell development. Wild type L2 and arrested *let-765(ok2058)* larvae carrying the *lin-48::gfp* reporter, *saIs14*. In wild type, the duct (du) can be seen curving away from the cell body towards the body wall (A, B). The position of the duct cell body is marked with an arrowhead. (C, D) An arrested *let-765(ok2058)* larva that is missing the duct cell. (E, F) Many *let-765(ok2058)* animals appear to have generated a duct cell, but the duct process does not extend to meet the body wall. Expression in the amphid neurons is not affected by mutations in *let-765*. Scale bar: 25 μm (A, C, and E) are Nomarski images merged with the GFP images in (B, D, and F).

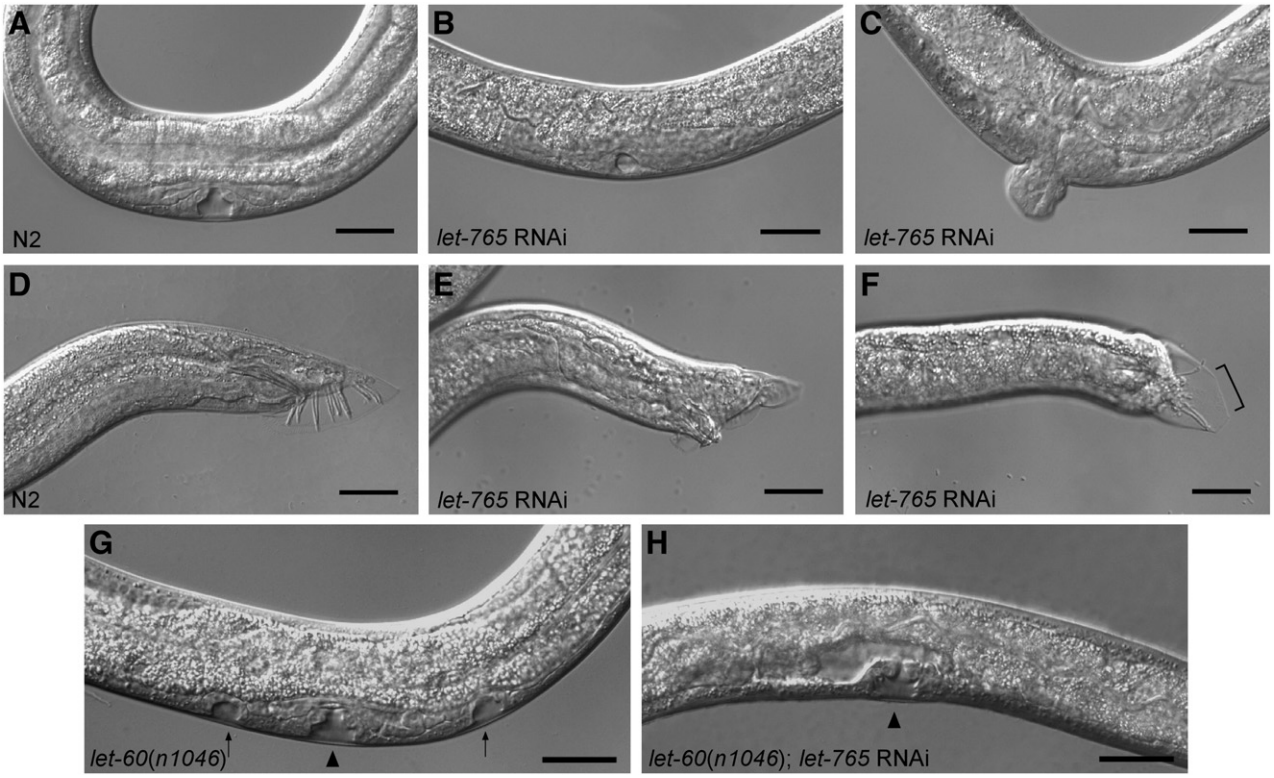

Fig. 4. *let-765* RNAi reveals a role for development of the vulva and male tail. (A–B) Nomarski images of L4 stage hermaphrodites, showing invagination of the vulval tissue. (A) wild type vulval cells having undergone the typical division pattern (B) *let-765* RNAi treated larva with a hypo-induced phenotype. (C) Adult *let-765* RNAi treated animal with a protruding vulva. All adult stage RNAi treated hermaphrodites exhibited the protruding vulva phenotype. (D–F) Adult male tails. (D) Wild type adult male. (E–F) Males with a reduction in *let-765* exhibit multiple tail defects including shortened spicules (E) and missing rays (F). The animal in (F) is facing ventrally with the location of missing rays bracketed. (G–H) *let-765* RNAi suppresses the Muv phenotype of *let-60*(*n1046*). An L4 stage *let-60*(*n1046*) animal (G) with a wild type vulva (arrowhead) flanked by two pseudovulvae (arrows) and (H) after exposure to *let-765* RNAi exhibits wild type vulval development. Scale bar: 25 μm.

role, we used RNAi to assess the effect of combining *let-765(rf)* with alleles that perturb EGFR/RAS pathway activity. Gain-of-function (gf) mutations in EGFR/RAS pathway genes produce a multivulva (Muv) phenotype where greater than three VPCs are induced; conversely reduction-of-function (rf) alleles lead to induction of less than three VPCs and a vulvaless (Vul) phenotype. We expected that if *let-765* promotes vulval induction, then a decrease in its activity would suppress a hyperactive pathway (gf) phenotype and would enhance a hypo-induced (rf) phenotype.

We initially focused on interactions with alleles of the ligand, LIN-3/EGF, and receptor, LET-23/EGFR. A partial loss of function allele of *lin-3*(*e1417*) has an incompletely penetrant Vul phenotype. We found that, in combination with *let-765* RNAi, vulval induction was further reduced in *lin-3*(*e1417)* mutants. Specifically, *lin-3*(*e1417*); *let-765* (RNAi) mutants were 100% vulvaless and exhibited a maximum of one cell induced compared to control *lin-3(e1417)* animals where 10% of animals exhibited normal levels of induction and developed a wild-type vulva (Table 1). In contrast, a multicopy transgene that overexpresses LIN-3 (Hill and Sternberg, 1992) results in a strong Muv phenotype. Reducing *let-765* activity could not suppress the hyperactive phenotype in these animals, indicating that *let-765* acts upstream of *lin-3*. To support this further, a ligand-independent gain of function allele of *let-23*/*egfr* (*sa62gf*) (Katz et al., 1996) was only mildly affected by *let-765* RNAi. The fact that the receptor was not sensitive to a reduction in *let-765* activity implies that LET-765 function is required upstream of LET-23, at or before the level of the LIN-3 ligand.

Additionally, we discovered that *let-765* RNAi was able to strongly suppress the Muv phenotype caused by a gain of function mutation in *let-60*/RAS (*n1046*) (Fig. 4G–H) (Beitel et al., 1990; Chang et al., 2000), and, to a lesser extent, from a temperature-sensitive RAS mutation, *let-60*(*ga89 gf*) (Eisenmann and Kim, 1997). Further to this, the over expression of an inducible wild-type MAP kinase, *mpk-1,* in combination with activated *mek-2* (Lackner and Kim, 1998) generates a Muv phenotype that was also suppressed by *let-765* RNAi (Table 1). Indeed, these results suggest that *let-765* may act downstream of the signaling cascade, in the VPCs, potentially affecting target gene expression. However, a clear interpretation is confounded by results from previous work which have demonstrated that the ectopic vulval induction in *let-60(n1046)* and *gaIs36* [hs-*mpk-1*; D*mek-2*] requires receptor activation by LIN-3/EGF (Chang et al., 2000; Dutt et al., 2004) and in gonad ablated *let-60(n1046)* or *gaIs36* animals, the induction index observed was similar to the level that we see with *let-765* RNAi. This would further support a role for *let-765* upstream of LET-23/EGFR, although, given the established role of *Drosophila* Sno downstream of EGFR signaling, it is reasonable to surmise that *let-765* may be required at multiple steps during vulval induction.

In an attempt to clarify the site(s) of *let-765* activity, we set out to examine potential interactions with downstream effectors of the pathway. RAS/MAPK signaling in the VPCs terminates with the transcription factors LIN-1/ETS and LIN-31. In the absence of MAPK phosphorylation activity, LIN-1 and LIN-31 form a dimer and inhibit vulval cell fates (Beitel et al., 1995; Tiensuu et al., 2005). Upon phosphorylation by MAPK, LIN-31 dissociates from LIN-1 and promotes vulval fates in the proximal VPCs, while inhibiting vulval fates in distal VPCs (Miller et al., 2000; Tan et al., 1998). Thus, loss-of-function mutations in *lin-1* are Muv and *lin-31* null mutants exhibit a mixed Muv/Vul phenotype (Miller et al., 2000). We determined that *let-765* RNAi did not affect *lin-1(e1777)* mutants and, furthermore, had only a mild effect on the level of vulval induction in *lin-31*(*n301*) and *lin-31*(*n1053*) mutants (Table 1). Additionally, a Wnt/β-catenin pathway promotes vulval cell fate specification in parallel to the

Table 1
let-765 promotes vulval induction.

Genotype	% muv[a]	% vul[b]	*n*	VPC induction[c] ± SE (*n*)
N2	0	0	>200	3.00 ± 0 (30)
rrf-3 (*pk1429*)	0	0	25	3.00 ± 0 (25)
rrf-3 (*pk1429*); *let-765* RNAi	0	30	27	2.44 ± 0.17 (27) **
lin-3(*e1417*)	0	90	50	0.86 ± 0.12 (50)
lin-3(*e1417*); *let-765* RNAi	0	100	52	0.26 ± 0.06 (52) ***
syIs1 [*lin-3*(+)extra]	100	0	50	5.20 ± 0.09 (20)
syIs1 [*lin-3*(+)extra];*let-765* RNAi	100	0	60	5.23 ± 0.11 (22)
let-23(*sa62*)	93	0	27	4.30 ± 0.12 (27)
let-23(*sa62*); *let-765* RNAi	89	7	27	3.85 ± 0.12 (27)
let-60(*n1046*)	98	0	91	4.29 ± 0.17 (28)
let-60(*n1046*); *let-765* RNAi	7	3	92	3.03 ± 0.13 (29) ***
let-60(*ga89*)	20	7	30	3.12 ± 0.09 (30)
let-60(*ga89*); *let-765* RNAi	3*	12	41	2.90 ± 0.06 (41)
[D*mek-2*(*gf*); hs-*mpk-1*][d]	81	0	27	4.17 ± 0.14 (27)
[D*mek-2*(*gf*); hs-*mpk-1*]; *let-765* RNAi[d]	57	0	21	3.48 ± 0.13 (21) **
lin-1(*e1777*)	100	0	55	4.70 ± 0.21 (10)
lin-1(*e1777*); *let-765* RNAi	100	0	40	4.47 ± 0.13 (15)
lin-31(*n1053*)	90	31	32	ND
lin-31(*n1053*); *let-765* RNAi	84	38	32	ND
lin-31(*n301*)	58	28	36	ND
lin-31(*n301*); *let-765* RNAi	37	34	65	ND
lin-12(*n137*)	100	0	45	6.00 ± 0 (18)
lin-12(*n137*); *let-765* RNAi	100	0	56	6.00 ± 0 (16)

Vulval induction was scored using Nomarski optics as described in Materials and methods.
**0.01 < *p* < 0.001, ***$p < 0.0001$; Data were compared with values from strains fed empty vector RNAi bacteria and statistical significance determined using a Mann–Whitney test or Fisher's Exact test.
[a] % Muv indicates the percentage of animals with ectopic induction (any of P(3, 4, or 8).p cell(s) induced).
[b] % Vul indicates the percentage of animals with an incomplete vulva (where P(5, 6, or 7).p are not induced).
[c] VPC induction was calculated as the average number of induced cells per animal. SE, standard error, *n*, number of animals scored, ND, not determined.
[d] Animals were heat shocked at 33 °C for 30 min at early L2, transferred to 23 °C and grown until L4.

EGFR/RAS pathway. The Muv phenotype caused by over activation of the Wnt pathway by *pry-1*/axin was not affected by *let-765* RNAi, nor was the hypo-induced (*rf*) phenotype of the *bar-1*/β-catenin allele (*ga80*) (Gleason et al., 2002) (data not shown).

The RAS/MAPK cascade triggers transcriptional activity in P6.p that specifies the 1° cell fate and includes the upregulation of DSL ligand expression. Three ligands have been identified as being relevant to vulval induction, encoded by the genes *lag-2*, *apx-1*, and *dsl-1*, which compose the lateral signal (Chen and Greenwald, 2004). In response to this signal, the neighboring cells, P5.p and P7.p, adopt 2° cell fates via the activation of LIN-12/Notch. A dominant gain of function allele of *lin-12*/*Notch (n137gf)* leads to a loss of the AC and all VPCs adopt the 2° fate, resulting in a Muv phenotype (Sternberg and Horvitz, 1989; Wang and Sternberg, 2001). This phenotype was not affected by *let-765* RNAi, demonstrating that LET-765 acts upstream of LIN-12/NOTCH activity, although there is still potential for an effect on the LIN-12 ligands which would be concealed by the dominant *n137gf* mutation. However, Chen and Greenwald (2004) have demonstrated that the expression of Notch ligands in P6.p is dependent on the activation of LET-23/EGFR signaling by LIN-3. Given that *let-765* RNAi leads to an apparent decrease in EGFR/RAS signaling at the level of LIN-3, we would expect that Notch ligand expression would also be reduced; as a result, any direct effect on ligand expression by reducing *let-765* activity would be clouded by the concurrent reduction in LIN-3 activity. When the expression of *lag-2::gfp* (*qIs56*) (Siegfried and Kimble, 2002)was examined (in *let-765* mutants and using *let-765* RNAi) we did not observe a detectable difference in expression pattern, in P6.p daughter cells or the distal tip cells, when compared to wild type (data not shown). Nevertheless, it is probable that LET-765 is required for the expression of one or more of the Notch ligands in P6.p, by analogy to the action of the *Drosophila* ortholog: Sno is required for the expression of the Notch ligand, Delta, downstream of EGFR for cone cell specification during eye development.

We attempted to quantify the proportion of *let-765* RNAi-treated larvae with defects in 2° cell fate specification by assessing the expression of cell specific markers. LIN-12 activity not only promotes the 2° cell fate in P5.p and P7.p, but represses EGFR/RAS pathway activity via the expression of *lip-1*, a MAPK phosphatase (Berset et al., 2001). In response to the lateral signal, *lip-1::gfp* expression is upregulated in P5.p and P7.p and reduced in P6.p. Using *let-765* RNAi, we assayed the expression of *lip-1::gfp* (*zhIs4*) at the early L3 stage. We found that in all animals examined, the expression pattern appeared wild type ($n = 33$) (Fig. S3). Another indicator of 2° cell fate is the transcription factor, *lin-11*, which is expressed in 2° cell lineages

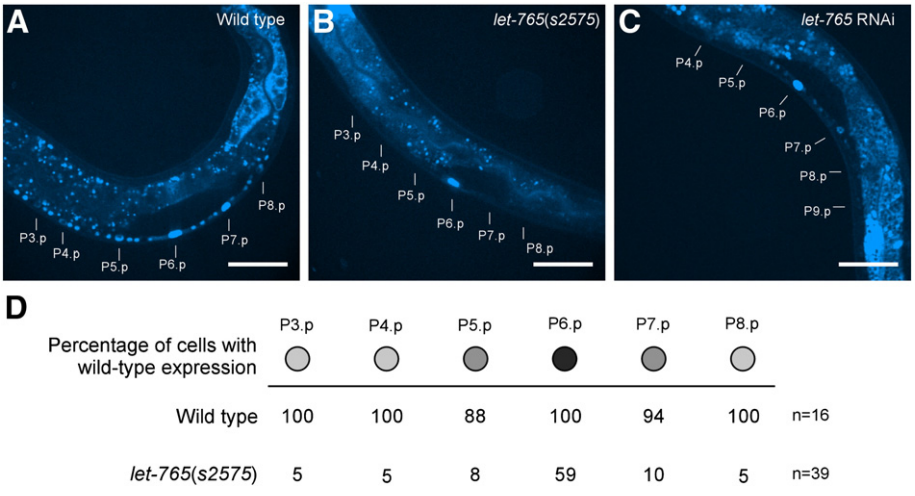

Fig. 5. *egl-17::cfp* reporter expression is lost in *let-765*(*s2575*) mutant larvae. Images (A–C) show reporter expression in mid-L2 stage hermaphrodites. (A) Wild type expression is highest in P5.p, P6.p, and P7.p, with lower expression in distal VPCs. In *let-765*(*s2575*) larvae (B), reporter expression was usually reduced or eliminated in all VPCs except P6.p. All animals were at early-mid L2 stage based on development of the gonad. Pn.p cells were not observed to divide in scored animals or in animals left for a further 24 h. (C) *let-765* RNAi led to a decrease in reporter expression in distal VPCs, but the effect was less penetrant than in *s2575* larvae. Scale bar: 20 μm. (D) Summary of the percentage of Pn.p cells that displayed a wild type level of *egl-17:cfp* expression, in wild type and *let-765*(*s2575*) L2 stage larvae. *n* = number of animals scored.

and in ventral uterine cells. When we analyzed the expression of *lin-11::gfp* (*syIs103*) in the presence of *let-765* RNAi, we found that the expression in 2° cell lineages was unchanged ($n=18$) (Fig. S4). All things considered, it is possible that *let-765* RNAi does not cause a sufficient decrease in gene expression to affect the output of these transcriptional reporters.

In summary, these data provide evidence that *let-765* positively regulates the EGFR/RAS pathway to promote vulval induction. LET-765 appears to function at or before the level of *lin-3*/*egf* since *let-765* RNAi cannot suppress the constitutive activity of the *let-23*(*gf*) allele, but can enhance a partial reduction in *lin-3*/*egf* activity. LET-765 may also function in the VPCs to promote the expression of DSL ligand(s); however, a definitive test of this activity has not been met at this point.

The s2575 mutation reduces egl-17 expression in VPCs

egl-17 acts as a reporter for *lin-3* activity as it is a primary transcriptional target of RAS signaling in the VPCs (Burdine et al., 1998). To obtain support for our genetic data, we examined how *let-765* affected the expression of *egl-17::cfp* (*arIs92*). The reporter is first expressed in the VPCs in mid L2 stage larvae, prior to the activation of LIN-12/NOTCH via lateral signaling. It exhibits a graded expression pattern that is brightest in P6.p and lower in flanking Pn.p cells (Dutt et al., 2004). Early in the L3 stage, Notch signaling leads to downregulation of EGFR/RAS activity in P5.p and P7.p, and thus reporter expression becomes restricted to P6.p. Since the *s2575* allele arrests during L2, we were able to examine EGFR/RAS activity, just prior to vulval induction, in a homozygous mutant background. Loss of *let-765* function mildly affected expression in P6.p; however, in 90% of animals ($n=39$), the signal was reduced or eliminated in the remaining VPCs (Fig. 5). *egl-17::cfp* expression was consistently visible in the M4 pharyngeal neuron, serving as a control for the presence of the reporter. Expression patterns were similar, though less penetrant, using *let-765* RNAi as compared to *let-765*(*s2575*) animals. In addition, during the L3 stage, *egl-17::cfp* expression was restricted to P6.p, where it was slightly reduced by *let-765* RNAi in a few cases. Similar results were obtained with and without *rrf-3* (*pk1426*). Overall, the loss of early reporter expression in P5.p, P7.p, and distal VPCs demonstrates that LET-765 is required for generating a wild-type pattern of *egl-17* expression. These results are consistent with the genetic data and in turn suggest that *let-765* is required for *lin-3* expression to reach a physiologically relevant level.

LET-765/NSH-1 is required for the synMuv phenotype

During vulval induction, the synMuv genes repress *lin-3* expression in the hyp7 hypodermal syncytium, thus restricting the action of the EGF signal and maintaining a wild-type pattern of cell fates (Cui et al., 2006a). In synMuv mutant animals *lin-3* is derepressed, activating the RAS/MAPK pathway, and resulting in ectopic VPC induction; it follows that a reduction in *lin-3* activity in hyp7 leads to suppression of the synMuv phenotype. Since the results from our first set of experiments implied that *let-765* is functionally required at the level of *lin-3* expression to promote RAS signaling, we chose to examine the effect of *let-765*(*rf*) on the synMuv phenotype.

let-765 RNAi completely suppressed the ectopic vulval induction conferred by the synMuv mutation *lin-15AB*(*n765*) (Clark et al., 1994; Huang et al., 1994), a complex locus that encodes both synMuv A and synMuv B activities. Moreover, 37% of *lin-15(n765)*; *let-765* RNAi larvae exhibited a vulvaless phenotype. To rule out specificity with the *lin-15* allele, we tested several other synMuv genotypes. Reducing *let-765* activity had a significant effect on vulval induction in most of the strains (Table 2). One notable exception was *lin-35*(*n745*); *lin-8* (*n111*) (Ferguson and Horvitz, 1989), in which *let-765* RNAi caused early larval arrest in all F1 animals. Experiments with other strains that carried *lin-8*(*n111*) resulted in synMuv suppression but no lethality, indicating that *let-765* functions in a common process with *lin-35*/Rb that is required for larval development and viability. In summary, *let-765* is genetically epistatic to synMuv mutations and appears to be required for the synMuv phenotype. These observations, together with the earlier finding that *let-765* affected EGFR/RAS activity upstream of receptor activation, suggest that LET-765 affects production of the LIN-3 ligand.

Table 2
The synMuv phenotype requires *let-765* activity.

Genotype	% muv[a]	% vul[b]	*n*	VPC induction[c] ± SE (*n*)
lin-15(*n765*)‡	100	0	190	5.94 ± 0.03 (57)
lin-15(*n765*); *let-765* RNAi‡	0	37	59	2.36 ± 0.16 (59) ***
lin-36(*n766*); *lin-15A*(*n767*)	100	0	114	5.82 ± 0.08 (22)
lin-36(*n766*); *lin-15A*(*n767*); *let-765* RNAi	10	6	115	3.57 ± 0.23 (21) ***
lin-8(*n111*); *lin-15B*(*n374*)	100	0	151	5.65 ± 0.10 (23)
lin-8(*n111*); *lin-15B*(*n374*); *let-765* RNAi	22	0	72	3.04 ± 0.04 (26) ***
lin-8(*n111*); *lin-9*(*n112*)	100	0	105	5.87 ± 0.07 (23)
lin-8(*n111*); *lin-9*(*n112*); *let-765* RNAi	76	9	35	4.29 ± 0.19 (35) ***
lin-53(*n833*); *lin-15A*(*n767*)	100	0	95	5.85 ± 0.06 (41)
lin-53(*n833*); *lin-15A*(*n767*); *let-765* RNAi	100	0	86	5.74 ± 0.08 (42)
lin-35(*n745*); *lin-8*(*n111*)	98	0	43	5.72 ± 0.13 (43)
lin-35(*n745*); *lin-8*(*n111*); *let-765* RNAi	n/a	n/a	many	L1 arrest

‡L4 animals were grown at 15 °C until reaching the adult stage and were then transferred to 23 °C.
***$p<0.0001$. Data were compared with values from strains fed empty vector RNAi bacteria and statistical significance determined using a Mann–Whitney test.
[a] % Muv indicates the percentage of animals with ectopic induction (any of P(3, 4, or 8).p cell(s) induced).
[b] % Vul indicates the percentage of animals with an incomplete vulva (where P(5, 6, or 7).p are not induced).
[c] VPC induction was calculated as the average number of VPCs induced per animal. SE, standard error, *n*, number of animals scored.

synMuvB phenotypes are not suppressed by reduced let-765/nsh-1activity

In addition to their repressor activity during vulval development, the synMuvB genes are required for transcriptional regulation during a number of other processes including cell division and proliferation (Boxem and van den Heuvel, 2001; Fay et al., 2002), germline versus somatic cell fate decisions (Kawasaki et al., 1998; Unhavaithaya et al., 2002), RNAi (Wang et al., 2005), and context-dependent transcriptional gene silencing (Tam phenotype) (Hsieh et al., 1999). A number of genes have been identified based on their ability to suppress ectopic vulval induction in synMuv mutants and many of these genes also suppress synMuvB phenotypes (Andersen et al., 2006; Cui et al., 2006b). Although *let-765* shares synMuv suppressor activity with this group, it did not affect synMuvB specific phenotypes.

mep-1 and *let-418*/Mi-2 both exhibit synMuvB activity and are required for restricting ectopic germ cell fates in the soma. Mutations in either gene result in a germline-like appearance of somatic cells and lethality (Unhavaithaya et al., 2002). Unlike other synMuv suppressor genes, *let-765* RNAi did not suppress the lethality of *mep-1*(*q660*) or *let-418*(*n3536*), or affect the cellular morphology. Furthermore, *let-765* RNAi did not cause de-silencing of a *let-858*::*gfp* transgene in the germline (Kelly and Fire, 1998) or affect ectopic expression of *lag-2*::*gfp* in the intestine (Poulin et al., 2005) (data not shown). From these results, we propose that *let-765* does not promote the adoption of ectopic germ cell fates in the soma, nor is it required for repressing transgene expression in the germline, and extend this to propose that *let-765* does not antagonize synMuvB transcriptional targets in these processes.

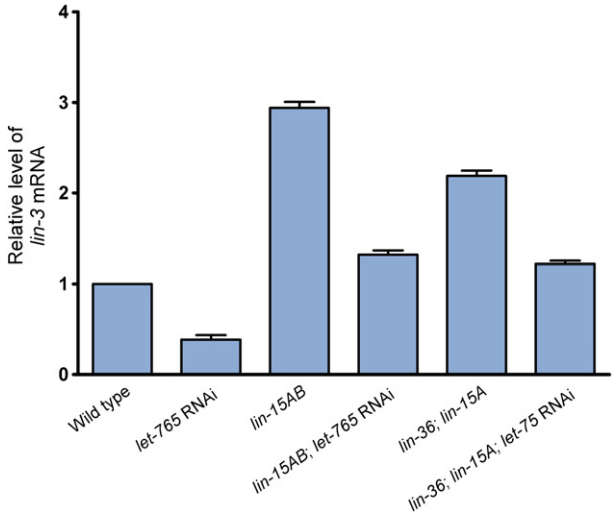

Fig. 6. Abundance of *lin-3* transcript is reduced in *let-765* RNAi treated animals. Real-time RT-PCR was performed on RNA from early L3 stage worms of the indicated genotypes using *rpl-19* as an internal reference. Mean values and standard deviations of relative *lin-3*/*rpl-19* ratios from three replicates are shown.

LET-765/NSH-1 regulates expression of lin-3/egf

Given the observation that *let-765* has a role in regulating *lin-3* activity and that *let-765* encodes a protein with homology to genes that are required for RNA metabolism and transcriptional regulation, we hypothesized that *let-765* may be involved in the regulation of *lin-3* transcription. Furthermore the repressive effect of synMuv gene function on *lin-3* transcription has been well demonstrated (Andersen and Horvitz, 2007; Andersen et al., 2008; Cui et al., 2006a) and forms the basis of the synMuv phenotype. Currently, the preferred method for analysis of the level of *lin-3* transcription is real-time RT-PCR as fluorescent reporters do not accurately reflect deregulation of *lin-3* expression (Cui et al., 2006a). We analyzed two synMuv mutant genotypes that were strongly affected by *let-765* RNAi, *lin-15AB*(*n765*) and *lin-36*(*n766*); *lin-15A*(*n767*), to determine whether suppression of their synMuv phenotype was a result of reduced *lin-3* transcription. qRT-PCR was used to measure the relative amount of *lin-3* mRNA present in control versus *let-765* RNAi treated strains, near the L2/L3 molt when induction occurs. In both synMuv genotypes tested, we found that the level of *lin-3* transcript decreased to approximately wild-type levels when *let-765* activity was reduced (Fig. 6). In addition, in wild-type animals treated with *let-765* RNAi, the level of *lin-3* mRNA decreased more than two-fold. These results indicate that *let-765* function is required for maintenance of efficient *lin-3* transcription. It should be noted that mRNA levels of other RAS pathway components and of synMuv genes were not significantly affected by *let-765* RNAi (Table S1). The suppression of the synMuv phenotype observed in RNAi treated animals likely results from a reduction in *lin-3* transcript levels and, as predicted by Andersen et al. (Andersen et al., 2008), the observed induction phenotype reflects the level of *lin-3* mRNA produced (*let-765* RNAi-treated synMuv mutants exhibit nearly wild-type induction).

Discussion

let-765 promotes gonad-dependent vulval induction as well as the synMuv phenotype

We have identified the essential gene, *let-765*, as F20H11.2/*nsh-1*, a DExD/H box helicase protein and demonstrated its role in promoting vulval induction by positively regulating *lin-3*/*egf* expression to maintain physiologically relevant levels of the ligand. Our genetic analyses, using *let-765* RNAi in concert with mutations in RAS pathway components, substantiates the proposed function for *let-765* in promoting vulval induction at the level of *lin-3* and, furthermore, confirms that *let-765* is required for RAS-dependent induction in the absence of a hyperactive RAS pathway. In addition to the reduced induction observed in *rrf-3*; *let-765* RNAi animals, we noted several interactions which suggest that *let-765* activity is required for expression of the gonad-dependent signal. First, in *lin-3*(*e1417*) mutants, where AC-specific expression of *lin-3* is reduced, *let-765* RNAi enhances the vulvaless phenotype; the percentage of *e1417* larvae with zero VPCs induced increased from 35% with empty vector control to 74% with *let-765* RNAi. Second, *let-60(n1046)* and *lin-15* (*n765*) strains exhibit suppression of ectopic induction, but also reduced induction of P(5-7).p when treated with *let-765* RNAi, again suggesting that the gonadal signal is affected. Several instances of reduced induction in proximal cells were also observed in *lin-36* (*n766*); *lin-15A*(*n767*) and *lin-8*(*n111*); *lin-9*(*n112*) animals. We would argue that the partial decrease in P(5-7).p induction reveals an effect on the gonad-dependent source of *lin-3*, which normally promotes the development of a wild-type vulva.

On the other hand, the ectopic induction displayed by synMuv mutants results from derepression of *lin-3* in hyp7 (Cui et al., 2006b; Myers and Greenwald, 2005). Our results demonstrate that synMuv strains treated with *let-765* RNAi display decreased ectopic induction as well as decreased "wild-type" vulval induction. As such, we propose that, in addition to suppressing the gonad-dependent inductive signal, *let-765* RNAi suppresses the gonad-independent (synMuv) activity from hyp7. Therefore, it is highly likely that LET-765 has a role in promoting the expression of *lin-3* in both the somatic gonad and hyp7. Nuclearly localized LET-765::GFP expression in these tissues at the time of induction is consistent with protein activity at these sites. In summary, it is feasible that *let-765* acts to positively regulate *lin-3* expression in multiple cell types and, where appropriate, consequently antagonizes the activity of the synMuv genes. Since the Muv phenotype in *let-60*(*gf*) animals is suppressed in the presence of wild-type synMuv proteins, we reason that LET-765 functions beyond simply blocking the repressive activity of synMuv genes. Instead we postulate that, in a synMuv mutant, *let-765* interacts with factors that promote *lin-3* activity while transcriptional repression is relieved.

The response to *let-765* RNAi in the examined synMuv strains reveals substantial variation in the interaction between *let-765*(*rf*) and some of the synMuv genotypes. For instance, synthetic lethality occurs when *let-765* levels are reduced in a *lin-35*(*n745*); *lin-8*(*n111*) mutant, which indicates that *let-765* and *lin-35* cooperate to promote larval development. *lin-35* is the *C. elegans* homolog of the mammalian tumour suppressor gene Rb, which interacts with the E2F and DP heterodimeric transcription factors to repress E2F target-gene expression. *lin-35*/Rb exhibits synthetic lethality with several other genes that include *pha-1*/FOXO, *ubc-18* (Mani and Fay, 2009), *psa-1*/SWI3 (Cui et al., 2004), and *slr-2* (Kirienko et al., 2008), revealing a requirement for *lin-35* in cell cycle control, cell proliferation, organ development, and fertility. Thus, it appears that LIN-35 operates redundantly in many processes, and as a result it is likely that, in *C. elegans*, the function of both LET-765 and LIN-35 converge on a common target in early development.

In contrast to *lin-35*, the failure of *let-765* RNAi to suppress ectopic vulval induction in *lin-53(n833)*; *lin-15A(n767)* indicates that derepression by the *lin-53* mutation does not rely on *let-765* activity. LIN-53 is the *C. elegans* homolog of the mammalian RbAp48 histone binding protein, which together with HDA-1/HDAC and LET-418/Mi-2 forms a Nucleosome Remodeling and Deacetylase (NuRD)-like complex. In *C. elegans,* LIN-53 has also been identified as part of the DRM complex, a multi-protein complex, which contains homologs of the *Drosophila* Myb-MuvB and DREAM complex components. The DRM and NuRD complexes are predicted to function separately to

repress vulval induction, implying that LIN-53 may be required at multiple steps in *lin-3* repression. In addition, the *n833* mutation causes ectopic vulval induction even when heterozygous, demonstrating potential dominant negative activity (Lu and Horvitz, 1998). Antagonism of LIN-53 by LET-765 may not be required for expression of *lin-3,* or, alternatively, the failure to suppress the synMuv phenotype by *let-765* RNAi may result from distinct sites of activity for *lin-53* and *let-765*.

let-765 phenotypes predict a role in other EGFR/RAS-mediated processes

The early larval arrest phenotype of *let-765* null mutants reveals a requirement for its activity in promoting osmoregulatory function and resembles the phenotype of strong mutations in EGFR/RAS pathway components. *let-60*/RAS plays a key role in the specification of the excretory duct cell; larvae missing this cell arrest at L1 and fill with fluid. Likewise, *let-765* null mutants are rod-like lethal and arrested larvae exhibit a loss or mis-specification of the excretory duct, predicted by the expression pattern of *lin-48::gfp.* Taken together, these results are consistent with a role for LET-765 in promoting viability through a common process with the EGFR/RAS pathway. Moreover, we can speculate that *let-765* may positively regulate *lin-3* expression to promote larval viability, in a manner similar to that used during vulval development. The *dpy-7p::*LET-765 transgene was able to rescue the lethality and osmoregulatory defects of *let-765* null mutants, implying that the hypodermis is an essential site of its activity. This differs from results obtained using mosaic analysis for *let-60*/RAS and *let-23*/EGFR, which indicate a focus of essential activity in the excretory duct and neurons, respectively (Koga and Ohshima, 1995; Yochem et al., 1997). As the hypodermis is intimately connected with the excretory system (and some of the nervous system), we could predict that *let-765* activity in hypodermal tissue is adequate for partial rescue of RAS defects. Alternatively, *let-765* may promote the expression of another product that positively regulates EGFR/RAS activity in the excretory and/or nervous system. In any case, *let-765* mutants exhibit other morphological defects in the excretory system that are not typical of previously described RAS pathway phenotypes and suggest a regulatory role for *let-765* in the development of this system beyond its potential effect on the RAS pathway.

In addition to vulval development and larval viability, we have discovered other *let-765* phenotypes that are similar to those seen in *lin-3* mutants. Rescued null mutants exhibit a completely sterile phenotype that is reminiscent of a loss of function in *lin-3* or *let-23* (Ferguson and Horvitz, 1985). Hermaphrodite ovulation is controlled by a RAS-independent process via LIN-3/EGF and LET-23/EGFR and IP3 signaling (Clandinin et al., 1998; McCarter et al., 1999). Secondly, male tail development is severely disrupted by a reduction in *let-765* function, leading to spicule defects, missing rays, and underdeveloped tails that may result from defective retraction of the hypodermal cells during morphogenesis. The spicule defects are consistent with an interaction with the EGFR/RAS pathway; however, the significant variation in phenotypes implies that LET-765 is involved in the development of other cell types in the tail. The loss of V6-derived rays in some *let-765* RNAi animals hints at a potential interaction with components regulating *pal-1*-dependent ray development (Zhang and Emmons, 2000, 2002). Overall, *let-765* appears to function in many EGFR/RAS pathway regulated cell fate decisions, but may also interact with other signaling pathways to promote distinct developmental processes.

let-765 and the regulation of Notch ligand expression

Given that *let-765* is the single *C. elegans* ortholog of *Drosophila sno* and that they are well conserved within the functional domains, it is reasonable to predict that they share a common molecular function and likely interact with similar genetic components. *sno* has been identified as a downstream player during inductive Notch signaling events, potentially through interactions with Su(H) (Majumdar et al., 1997; Nagel et al., 2001). We have yet to identify *let-765* phenotypes that specifically implicate an interaction with Notch signaling, however, the phenotype could be too subtle, or the observation of an interaction may require additional activities to be compromised. *Drosophila* Sno also has a well defined role in promoting the expression of the Notch ligand, Dl, downstream of EGFR during cone cell specification where it interacts with transcriptional regulatory proteins to interrupt repression of Dl by Su(H) and the co-repressor SMRTER (Nagaraj and Banerjee, 2007; Tsuda et al., 2002). The strong parallel between the sequential action of EGFR/RAS and Notch signaling during development of the *Drosophila* eye and the *C. elegans* vulva has been nicely illustrated (Sundaram, 2005; Voas and Rebay, 2004). Indeed, our finding that *let-765* is required during vulval induction poses an attractive possibility that Sno activity is conserved during vulva development and this led us to hypothesize that *let-765* could function in the VPCs at a terminal step in the EGFR/RAS pathway to promote the expression of DSL ligand(s). Nonetheless, we have gathered considerable support for a role for LET-765 in promoting the expression of *lin-3*/*egf*, which complicates any interpretation of the dependence of DSL gene expression on *let-765* activity. This is due to the fact that the expression of DSL ligands in P6.p is directly regulated by LIN-3 and the EGFR/RAS pathway; in a *lin-3 (e1417)* mutant, transcriptional reporter activity from DSL genes is abolished (Chen and Greenwald, 2004). Since *lin-3* expression is reduced in response to a decrease in *let-765* activity, it follows that the expression of DSL genes would also be affected and a measure of any direct contribution by LET-765 on DSL gene expression in P6.p would be impossible to differentiate. Future biochemical investigations may supply a definitive answer to this question, and could provide clues to the nature of transcriptional complex(es) that function in P6.p.

let-765 RNAi primarily affected the induction of P5.p and P7.p, as completely vulvaless animals were only observed using a sensitized genetic background. However this could be due to a number of factors. Most importantly, the L3 larvae that we use to score vulval induction are escapers, and therefore do not represent a true loss of function. We should recognize that a complete loss of *lin-3* expression, like *let-765*, leads to larval lethality, so our results will not reflect a complete loss of *lin-3* expression in the AC. Additionally, we cannot rule out a contribution by other proteins, as LET-765 likely interacts with additional components to promote *lin-3* expression and, furthermore, may do so by affecting the activity of intermediary products.

A potential role for let-765-mediated gene expression in the anchor cell

The details surrounding the activation of *lin-3* expression in the anchor cell (AC) have not yet been fully realized; however several studies have lent some insight into a potential mechanism. The LIN-12/Notch pathway is required for determining the AC fate at the L2 stage via a well studied lateral inhibitory process (Greenwald et al., 1983; Seydoux and Greenwald, 1989). Following specification, the *lag-2*/DSL ligand is expressed in the AC and functions to induce cells in the neighboring somatic gonad to acquire the π cell fate (Newman et al., 2000); the π cells are necessary for the formation of the vulval-uterine connection subsequent to vulval induction. Thus, the AC expression of *lag-2* overlaps with that of *lin-3* and it has been suggested that LIN-12/Notch signaling may be involved in the establishment of *lin-3* expression in the AC, potentially through the action of the transcription factor, *hlh-2* (Hwang and Sternberg, 2004). *C. elegans* HLH-2 is a basic helix–loop–helix (bHLH) transcription factor and the E protein/Daughterless homolog. HLH-2 has been shown to bind E box enhancer elements in the *lin-3* promoter and its activity is required for anchor cell expression of *lin-3* (Hwang and Sternberg, 2004) and *lag-2* (Karp and Greenwald, 2003). bHLH

proteins are known to function downstream of Notch signaling during specification of cell fates in *Drosophila*; consequently HLH-2 may provide a similar function during vulval development in *C. elegans*.

An overlapping requirement of *let-765* and *hlh-2* for *lin-3* expression during vulval induction suggests potential for collaboration in promoting *lin-3* transcription. Further, although *let-765* RNAi animals do not exhibit defects in specification of the AC or the distal tip cells (data not shown), as is seen in *hlh-2* RNAi (Karp and Greenwald, 2004), the documented activity of *Drosophila* Sno downstream of Notch signaling raises the question of whether a conservation of function (in this context) exists between LET-765 and Sno, to promote the expression of Notch target genes. With this in mind, the occasional loss of π cell expression in *lin-11::gfp*; *let-765* RNAi animals is suggestive of an additional function for *let-765* in the AC. Future investigations into a possible AC-specific role for *let-765* may lend insight into additional overlap between Notch and EGFR/RAS signaling and into the action of *strawberry notch* proteins in general.

DExD/H proteins and gene expression

The role of DExD/H box proteins in RNA processing and translation have been well established by studies both in vitro (Fairman et al., 2004; Jankowsky et al., 2001; Lai et al., 2008; Lee et al., 2008; Schwer, 2001) and from model systems (Johnstone and Lasko, 2004; Rajyaguru and Parker, 2009; Salinas et al., 2007; Tomancak et al., 1998). However, only recently are we starting to learn about their role in transcriptional regulation. Human RNA helicase A (RHA) is a homolog of the *Drosophila* gene *maleless*(*mle*), which is required for transcriptional repression and dosage compensation of the X chromosome. Human RHA has been shown to bind CREB binding protein (CBP) and RNA Pol II to activate CBP-dependent transcription through a transactivation domain (MTAD) that has also been identified in *Drosophila mle*. Furthermore, the MLE protein can recruit RNA Pol II to target genes via MTAD. Additionally, RHA has been shown to facilitate an interaction between the tumor suppressor BRCA1 and RNA Pol II, through a region of the protein distinct from that of MTAD. The *C. elegans* homolog, *rha-1*, is required for transcriptional silencing in the germline, but an activator function is as of yet unidentified. Another well studied DEAD-box helicase, p68 and its homolog p72, have been shown to possess both transcriptional activator and repressor functions. Both proteins interact with estrogen receptor α (ERα) and, like RHA, interact with CBP/p300 and RNA Pol II, suggesting a common "bridging" function may exist for some DExD/H box proteins. More recently, p68/p72 have been shown to immunoprecipitate with HDAC1, and promoter specific transcriptional repressor activity has been demonstrated, highlighting the potential diversity of DExD/H box helicase function.

The faithful reproduction of complex developmental programs relies on the precise spatio-temporal expression of intercellular signals, promoting or modifying the expression of downstream target genes. Often it is the action of sequence-specific DNA binding proteins that directly regulate gene expression. However, a role for additional co-activator proteins in an interaction with the basal transcriptional machinery has been identified that enables the integration of diverse regulatory inputs at specific promoters. Subsequent to the establishment of an active chromatin structure by remodeling proteins that include SWI/SNF homologs and histone modification proteins, the recruitment of RNA Pol II and general transcription factors commences in concert with the Mediator complex—a multi-subunit complex that integrates signals from activator/repressor cofactors and the basal transcriptional machinery (Emerson, 2002). It has been suggested that Mediator subunit composition, together with cofactor interactions and their substrate specificity, could lead to distinct activities at different promoters. The predicted role for DExD/H box proteins, and more specifically *strawberry notch* family proteins, in transcriptional regulation, together with our current data that demonstrates a role for LET-765 in positively regulating *lin-3* expression, suggests that LET-765 likely promotes the expression of target genes via an interaction with co-activator proteins or complexes. More specifically, our data points to a role in either promoting transcription or maintaining the stability of newly synthesized mRNA. Thus, we have identified a new positive regulator of *lin-3*/*egf* expression and a novel role for a *strawberry notch* family protein. Furthermore, the nearly ubiquitous expression pattern and variety of phenotypes generated by a loss or reduction in *let-765* function is consistent with a global role for LET-765 in promoting gene expression through multiple interactions or pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.03.004.

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